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HISTOGENESIS BY CELLS FROM EMBRYONIC AND HATCHED CHICKS IN GIANT, PLATE-LIKE AGGREGATES CULTURED ON A POROUS MATRIX

KRYSTYNA D. ANSEVIN AND BINIE V. LIPPS

Department of Biology and Department of Chemical Engineering, Rice University, Houston, Texas

It was demonstrated first by Moscona and Moscona (1952) that monodispersed cells of amniote embryos can reaggregate and then build histotypic structures. With a more refined technique, Moscona showed that these capacities are efficient in cells of 7–10 day old chick embryos but decline with the age of the embryo. In his experimental system retinal cells from 7-day chick embryos formed aggregates of 0.3–1 mm in diameter but cells from 19-day-old fetal chicks failed to aggregate altogether (Moscona, 1961, 1962). Lung cells from 16–18 day old embryos aggregated, but showed poor or abnormal histogenesis (Grover, 1961). Even when implanted on chorioallantoic membrane, late fetal or postnatal mouse cells showed limited aggregation and in only a few cases a trace of histogenesis (Ansevin and Buchsbaum, 1962). These results suggested that the capacity to reaggregate and to reconstruct the tissue of origin might be limited to less advanced embryonic stages. This assumption gained support from Kuroda's work (1968) in which the aggregation of cells from older embryos was enhanced by medium that had been conditioned by cells of the same organ from younger developmental stages. Previous experiments with cell aggregation in other laboratories resulted in rapid histotypic reconstruction; however, the aggregates were not maintained in culture for more than 4–6 days.

This report reinvestigates the dependence of aggregation and histogenesis on the age of the cells by means of a new technique, the tissue plate culture (Ansevin and Lipps, 1973). The technique promotes formation of gigantic aggregates (tissue plates) from cells of more advanced developmental stages and sustains their organotypic organization for a longer time than usually is feasible with conventional methods of organ culture.

Materials and Methods

Suspensions were prepared from cells of 14- or 18-day-old chick embryos, and, also, from newly hatched, 2-day-old and 4-day-old chicks. Kidneys or lungs from several animals were pooled in Tyrode salt solution, minced with fine knives and transferred to test tubes. After several rinses and a 10-minute incubation in Calcium-and-Magnesium-free Tyrode (CMF) the tissue preparations were washed with a 1–3% solution of trypsin (Nutritional Biochemicals) in CMF and incubated in a fresh change of trypsin solution for 20 minutes at 38 degrees Centigrade. After that, the tissues were transferred to the culture medium and dispersed by in and out pipeting with a capillary pipet. The preparation was left for several minutes for large undispersed tissue fragments to settle by gravity. The supernatant was transferred to another tube and further dispersed by repeated pipeting. This was followed by brief centrifugation at 20 *g* to remove undispersed tissue fragments. After this treatment it was found that the supernatant suspension consisted predominantly of single cells among which cell clumps of varying sizes and varying degrees of compactness were present. More culture medium was then added to the sedimented cells and the process of dispersion and centrifugation was repeated several times. Finally, all the supernatants were pooled and a sample drop again was examined under the microscope. This preparation was then centrifuged at 300 *g* for 15 minutes. After removing the supernatant, the compacted cells were resuspended in several drops of fresh culture medium and placed on top of a porous matrix in plastic Falcon petri dishes 50 mm in diameter. A piece of phenolic sponge—available from many florists—("Oasis," Smithers Co., P.O. Box 118, Kent, Ohio) was used as a matrix for the cells because of its hydrophilicity and the facility with which it could be sectioned on the microtome. The sponge was washed, cut into circles roughly 10 × 3 mm, autoclaved, and presoaked in culture medium. The culture medium consisted of 44% NCTC 135, GIBCO, 44% Tyrode solution, 10% fetal calf serum, 2% chick embryo extract (prepared in this laboratory), 50 units of penicillin, and 50 micrograms of streptomycin per milliliter of the final medium. After the cultures had stood undisturbed for 1–2 hours, 1.5 ml culture medium was added. After the next 48 hours the petri dishes were placed on a Bellco tissue culture rocker operating at the speed of 4 tips per minute throughout the period of cultivation. The cultures were incubated at 38° C in an atmosphere of 95% air and 5% CO_2. The culture medium was changed three times a week. The period of cultivation varied from 0 time (control for aggregation and histogenesis) to 4 weeks. It was followed by fixation of the culture-substrate complex in Zenker fluid, embedding in paraffin, and sectioning at 5 microns either parallel or perpendicular to the surface of the substrate. For staining, the Rapid One-Step methods of Mallory-Heidenhein (according to Humason, 1967) were used.

Although our dispersion procedure yielded cell suspensions in which the integrity of the organ-specific structures (nephric or bronchial tubules) was destroyed, one might suspect that the presence of cell clumps in addition to single cells could have aided reconstructive histogenesis. In order to test whether completely monodispersed cells of the same stages are also capable of undergoing histogenesis, suspensions of exclusively single cells and 2-cell clumps were prepared. This was done by filtering trypsinized suspensions through a Swinny filter assembly with

two layers of paper: one layer of "Kimwipe" (Kimberly-Clark Co., Neenah, Wisconsin) and one layer of Japanese "Mullberry drawing paper" (obtained through Andrews/Nelson, Whitehead, New York). These brands of paper gave the most satisfactory results in extensive series of tests of various kinds of paper. Immediately following filtration, only single cells and 2-cell "clumps" could be detected, and decrease in cell population was relatively insignificant. The cells from these suspensions were sedimented by centrifugation and otherwise prepared into cultures as described above for less complete cell dispersions.

Two different types of controls were used in this investigation. One type of controls involved cultures fixed immediately after preparation. This was used for determining the condition of dispersed cells in each experiment before aggregation and histogenesis occurred.

In controls of a second type, dispersed cells were cultured on discs of Millipore filter (assumed to be a two-dimensional, porous substrate) floating on top of the culture medium in Falcon dishes. Only embryonic cells from lung and kidney were used in this type of experiment.

Results

In embryonic and post-embryonic tissues, the cells coalesced extensively in the form of plate-like aggregates that frequently reached about 1 cm in diameter. However, the thickness of the plates never seemed to exceed 0.5 mm.

Kidney on the sponge matrix

When single cell and cell clump suspensions were used, there was no appreciable difference between the extent of aggregation of kidney cells of the following ages: 14-, 18-, 21-days. Histogenesis in plates of cells from 18-day-old chick embryos was as good as that from 14-day-old embryos. The epithelial structures formed ranged from cysts to long cylinders randomly arranged (Figs. 2, 3). Histogenesis in kidney cells from hatched chicks was more limited; nevertheless, tubular structures occurred as shown in Figure 4. These structures were not present in cultures fixed immediately after preparation. The cultures persisted in unchanged condition for 2–3 weeks; afterwards, there was a gradual increase in the amount of connective tissue (Fig. 5), followed by a slow disorganization of epithelial structures. Nevertheless, some tubules persisted in the cell plates for a month or longer. Fully monodispersed cells from 14- and 18-day embryos and from newly hatched chicks aggregated as large tissue plates in which kidney tubules were abundant. For reasons not understood at present, mortality among cells from 2- to 4-day-old chicks was very high. This factor and the existence of an exceedingly high contamination of post-embryonic cells with erythrocytes were very likely responsible for a decline in the formation of large tissue plates by kidney cells of these post-embryonic stages. Small aggregates still formed. Some of them were built of completely solid tissues and were of sufficient size that a tendency to restore histological organization could still be revealed in them by the presence of occasional cystic or tubular structures which showed more or less distinct morphology. Because of this result, we felt that the technique, rather than intrinsic cellular limitations influenced aggregation and histogenesis in postembryonic cells in an

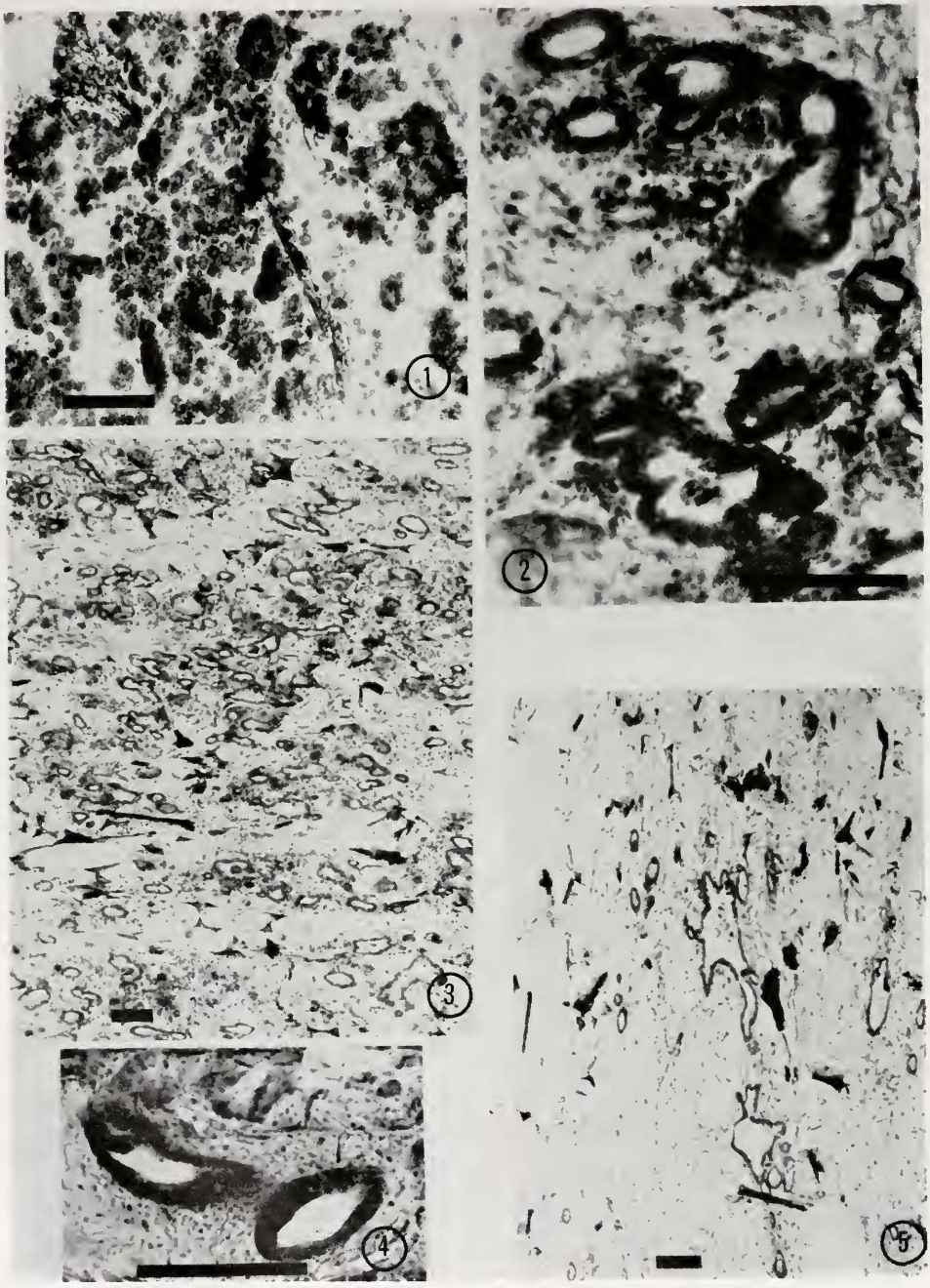

FIGURE 1. Control culture fixed immediately after preparation: Since the cells had been compacted by centrifugation and a certain time had elapsed until the culture was set up, cell aggregation is already under way. Scale bar equals 150 μ.

adverse manner. We are presently attempting to adapt the tissue plate technique so that post-embryonic cell viability would be increased, thus offering better opportunities for aggregation.

Lung on the sponge matrix

With single cells and cell-clump suspensions, extensive aggregation occurred with cells of 14-, 18-day embryos and, also, of newly hatched chicks. Histogenesis was excellent in plates of 14-day-old lung cells: bronchial tubules (Fig. 6) and cartilaginous rods were abundant. There was a quantitative decrease in reconstruction of tubules in plates of 18-day-old cells (Fig. 7), but no evident decrease in differentiation of cartilage. In plates from lung cells of hatched chicks, bronchial epithelium reconstructed tubules very rarely (Fig. 8); in several cases, capillaries with differentiated erythrocytes were found in these cultures (Fig. 10) and cartilagious rods or blocks were still occasionally present (Fig. 9).

The appearance of cells in the lung cultures suggested an insufficiency of a factor that would sustain the differentiated condition of the bronchial cells. After about a week of cultivation, epithelial cells in the tubules flattened, thus losing their typical morphology; after several more days bronchial epithelium degenerated and disintegrated while other types of cells still looked healthy and could survive much longer.

A single experiment with fully monodispersed cell suspensions of either 14- or 18-day embryos, confirmed the results described for the single cell, cell-clump suspensions. Extensive tissue plates formed which showed abundant bronchial tubules and nodules of cartilage. However, at the post-embryonic stages (immediately after hatching, 2-day and 4-day chicks) the proportion of erythrocytes in the sedimented material was very high and mortality of cells was considerable. As a result, solid tissue formed only as small aggregates, which in most cases were much too small to contain any tubular structure. Nevertheless, in a few instances a segment of a tubule lined with a healthy, bronchial epithelium was observed in cells from 2-day-old chicks.

Kidney and lung cells on Millipore filters

Cells of both organs reaggregated to some extent but did not show reorganization into tissue-specific structures (Fig. 11).

Discussion

The present study is providing new information concerning age dependence of cell aggregation and reconstructive histogenesis; this has been possible because the problem has been reinvestigated with the help of the tissue plate technique

Figure 2. Tubules reconstructed in the tissue-plate prepared from 14-day-old embryonic kidney after 6 days of cultivation. Scale bar equals 300 μ.

Figure 3. A small fragment (about 2 mm long) of a tissue-plate prepared from 18-day-old embryonic kidney after 6 days *in vitro*. Scale bar equals 70 μ.

Figure 4. Nephric tubules in the tissue plate reconstructed by dispersed kidney cells of the hatched chick. Six days of cultivation. Scale bar equals 300 μ.

Figure 5. A culture similar to (3) but cultivated for 3 weeks. Scale bar equals 70 μ.

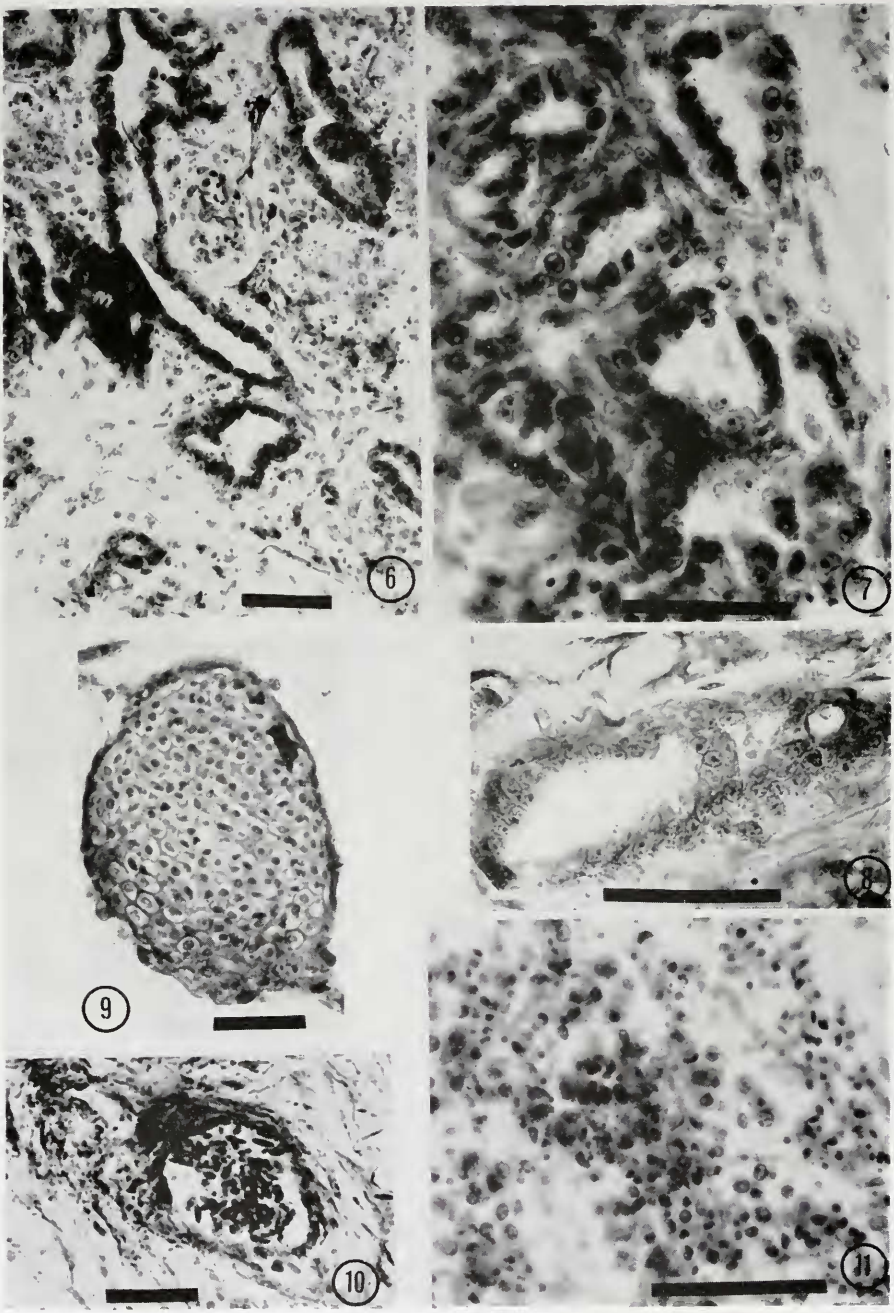

FIGURE 6. Bronchial tubules in the tissue-plate-culture prepared from 14-day-old embryonic lung. Six days of cultivation. Scale bar equals 150 μ.

(Ansevin and Lipps, 1973). This culture technique was developed by combining several older methods, such as cell aggregation and organ culture, with more recent techniques that utilize three-dimensional porous matrices as substrates for cells. By this means, the tissue plate culture technique overcomes several limitations inherent in each of the separate techniques. The application of a three-dimensional, porous matrix for the cells appears to promote their aggregation and subsequent histogenesis considerably more than do culture conditions which encourage formation of spherical aggregates. With the three-dimensional matrix, plate aggregates of relatively gigantic size are formed: about 10 mm in diameter and 0.3–0.5 mm in thickness. Spherical aggregates of embryonic cells obtained with previous methods, attained an average diameter close to 0.5 mm (Moscona, 1962) or 0.3 mm (Kuroda, 1968). Because considerably more cells are involved in formation of plate aggregates, their reconstructive histogenesis is superior to that of small spherical aggregates, a result consistent with the finding of Jones and Elsdale (1963) who determined that small size of aggregates was a limiting factor in their differentiation. In the present experiments, histogenesis occurred only in plate aggregates cultivated on three-dimensional porous substrates; cells cultured on Millipore filters aggregated but remained unorganized. We tentatively interpret this difference by assuming that convective circulation of medium is much less in three-dimensional substrates than around a thin Millipore filter and thus the products of cell interactions may remain at sufficient concentrations in the proximity of plate aggregates to promote their histogenesis.

The decline of aggregation and histogenesis with the age of the cell donor is much slower for plate aggregates cultivated on porous matrices than for spherical aggregates suspended in culture medium. In the experiments of Moscona (1962) and Kuroda (1968), cells from 18-day chick embryos essentially failed to aggregate; histogenesis was no longer present in aggregates formed by cells from 16-day embryos. In the conditions of the tissue plate culture, aggregation and histogenesis are excellent in cells of 18-day embryos; even at the immediately post-hatching stage, decline in aggregative capacities (of kidney cells) is not apparent and varying degrees of histogenesis (dependent on the type of tissue) are still accomplished. It was observed in the present experiments that cells of more advanced stages need longer time for reconstruction of tissue structures; this work is currently under progress.

To our knowledge, no comparable degree of aggregation or histogenesis was previously reported for cells of pre-hatching and post-embryonic chick stages or for similar developmental stages of mammals, although adult frog liver cells were shown to aggregate and exhibit histogenesis (Ansevin, 1964). The fact that fully mono-

FIGURE 7. Similar tubules in the plate-aggregate of 18-day-old embryonic chick. Four days of cultivation. Scale bar equals 150 μ.

FIGURE 8. A tubule in the tissue plate prepared from lung cells of hatched chick of cultivation. Six days of cultivation. Scale bar equals 300 μ.

FIGURE 9. Cartilage nodule differentiated in a week-old tissue plate prepared from lung cells of hatched chick. Twenty-one days of cultivation. Scale bar equals 150 μ.

FIGURE 10. A capillary-like structure with erythrocytes that is sometimes encountered in tissue-plate prepared from hatched chick lung cells. Seven days of cultivation. Scale bar equals 150 μ.

FIGURE 11. Lung-cells of 18-day-old chick embryo cultivated on Millipore filter for 4 days. Scale bar squals 300 μ.

dispersed cells from 4-day chicks can still occasionally form compact aggregates and, that these aggregates of post-embryonic cells, which are only semi-solid, appear to be highly contaminated with erythrocytes suggest that an intrinsic cell capacity for aggregation is not yet lost at young post-embryonic stages. Thus, it is possible that improved culture techniques would eliminate erythrocytes from cell suspensions and could decrease mortality of post-embryonic cells during preparation of the cultures, to permit further progress in the area of aggregation of post-embryonic cells. Since small size of aggregates in itself would be a factor hindering histogenesis (Elsdale and Jones, 1963), and, furthermore, since more solid and slightly larger aggregates of post-embryonic cells did show traces of histogenesis, it is likely that the low degree of histogenesis in the postembryonic cell cultures (following complete monodispersion) was again more of an expression of inadequate culture conditions than of inherent limitations in the post-embryonic cells. One may hope that this process could be evoked also in "older" cells by proper culture conditions.

This study also casts some light on the relationship between monodispersion of cells and their capacities to undergo subsequent aggregation and histogenesis. With cells of younger embryos (14 days) and even of those at 18 days of incubation differences between partly dispersed (including histologically structureless cell clumps besides single cells) and completely monodispersed cell suspensions were not obvious since both types of suspensions formed extensive tissue plates which were abundant in tubular and cystic structures. However, high mortality of fully monodispersed cells from newly hatched chicks was not evident in cultures which had been prepared with incompletely monodispersed suspensions; also, the former formed only small aggregates and showed only traces of histogenesis, while the latter were still able to aggregate into large tissue plates in which histological structures, although infrequent, were much better differentiated. This difference might be interpreted in general terms as a loss of a histogenetic factor from the cell surface during dispersion; a factor which would occur in larger quantities in embryonic than in post-embryonic cells. The presence of such factors has been determined in sponges (Humphreys, 1965) and strongly implied by Moscona's (1962) experiments with warm-blooded vertebrate cells.

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Summary

Tissue plate cultures on a sponge matrix were prepared from kidney or lung cells of 14- or 18-day chick embryos, of newly hatched chicks, and of chicks 2 or 4 days old. Two types of suspension were prepared from these cells and used for the cultures: *suspension 1* contained single cells and cell clumps of varying size and degree of compactness, but free from organized histological structures; *suspension 2* contained single cells and 2-cell clumps exclusively. In the tissue plate cultures on the three-dimensional substrate both kidney and lung cells at both embryonic stages formed giant, plate-like aggregates from either type of suspension; histogenesis in these aggregates was excellent. Kidney cells of newly hatched

chicks were also able to reaggregate into extensive tissue plates from either type of suspension; reconstructed tubules were still present in these cultures although considerably less abundant. Lung cells from newly hatched chicks formed large tissue plates only from *suspension 1;* occasionally a bronchial tubule, a cartilage nodule or a capillary-like structure was found in the plates. *Suspension 2* of lung cells from newly hatched chicks resulted in formation of small aggregates with rare indications of a histogenetic process. The same result was true for both kidney and lung cells in *suspension 2* from 2- and 4-day-old chicks. The possibility is discussed that in these experiments technical reasons involved in preparation of the cultures were largely responsible for the apparent decline in aggregation and histogenesis of post-embryonic cells. In control experiments embryonic cells cultivated on Millipore filters ("two-dimensional," porous matrices) did not form extensive tissue plates or develop histological organization.

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